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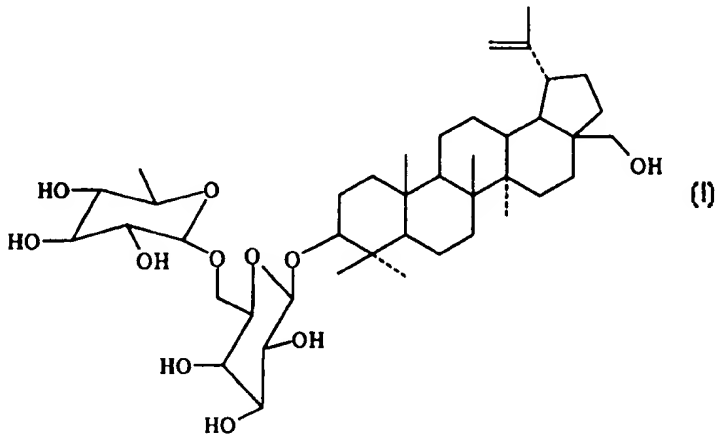
(54) Title: NOVEL TRITERPENE GLYCOSIDE COMPOUND, PROCESS FOR PREPARATION THEREOF AND ANTI-CANCER COMPOSITION CONTAINING THE SAME

(57) Abstract

The present invention relates to a triterpene glycoside compound represented by formula (I), which is extracted and isolated from Pulsatillae radix and has an anti-cancer activity, a process for preparation thereof, and an anti-cancer composition comprising as an active ingredient the compound of formula (I) or an extract which is obtained during the procedure for isolation of the compound of formula (I).



(I)

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NOVEL TRITERPENE GLYCOSIDE COMPOUND, PROCESS FOR PREPARATION THEREOF AND ANTI-CANCER COMPOSITION CONTAINING THE SAME

TECHNICAL FIELD

The present invention relates to a novel triterpene glycoside compound isolated from Pulsatillae radix, a process for preparation thereof including isolation from Pulsatillae radix and purification, and an anti-cancer composition containing as an active ingredient the triterpene glycoside compound or an extract which is obtained during the procedure for isolation of the glycoside compound.

BACKGROUND ART

Pulsatilla koreana Nakai is classified as a perennial herbaceous plant belonging to Ranunculaceae and is native plant ocurring at hillock and around the field in Korea. In the field of Chinese medicine, dry radix of *Pulsatilla koreana* Nakai has been called Pulsatillae radix and used as antipyretic, detoxicating and blood cleaning agents. Major components which have been isolated from this plant up to date include hederagenin glycosides such as akebioside St_h, cauloside E, etc. In addition to hederagenin glycoside components, plants belonging to Pulsatilla, including *Pulsatilla koreana* Nakai, commonly contain ranunculin, protoanemonine, anemonine, etc. Among these components, it has been reported that protoanemonine exhibits mitotic toxicity [see, Vonderbank et al.: Pharmazie 5, 210 (1950)]. In addition, Kim, et al. [see, S.Y. Kim, S.B. Kim, Journal of Korean Cancer Society, 26, 959-963 (1995)] have reported that the extract of Pulsatillae radix exhibits a potent cytotoxic activity (ID_{50} value 0.14-1.04$\mu g/m\ell$) against 10 kinds or more of cancer cell lines including A549, SUN cells, etc.

Although it has been known that the extract of Pulsatillae radix exhibits an anti-cancer activity as mentioned above, which component of the extract shows an anti-cancer activity has not yet been disclosed, and any active compnent having anti-cancer activity has not been isolated as yet.

Thus, the present inventors have extensively studied to isolate the active ingredient showing anti-cancer activity from the extract of Pulsatillae radix and identified that a certain triterpene glycoside compound isolated by treating Pulsatillae radix according to the method specifically described below shows a potent anti-cancer activity. Thus, we have completed the present invention.

DISCLOSURE OF THE INVENTION

Accordingly, the object of the present invention is to provide a novel triterpene glycoside compound showing an anti-cancer activity, which is isolated from the extract of Pulsatillae radix by a specific isolation and purification method.

Another object of the present invention is to provide a process for isolating a novel triterpene glycoside compound having anti-cancer activity from Pulsatillae radix.

In addition, the present invention also relates to an anti-cancer composition which contains as the active ingredient the novel triterpene glycoside compound isolated from Pulsatillae radix.

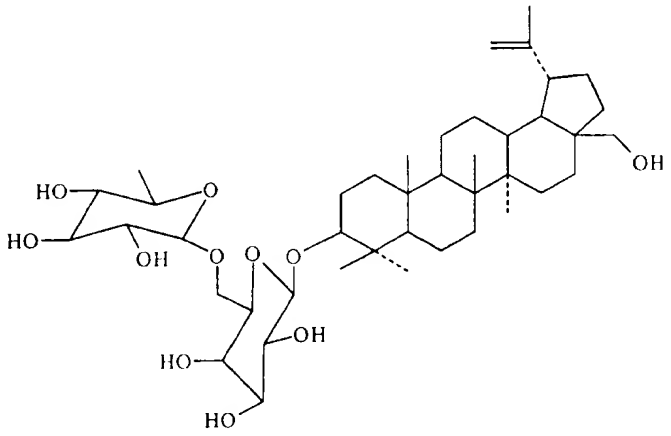
BRIEF DESCRIPTION OF DRAWINGS

For a thorough understanding of the nature and objects of the invention, reference should be made to the following detailed description taken in connection with the accompanying drawing in which:

Figure 1 shows the result of silica gel thin layer chromatography of the extract A and the extract B obtained in Example 1 and the pure material (I) obtained in Example 2 [lane 1: pure material (I), lane 2: extract B, lane 3: extract A].

BEST MODE FOR CARRYING OUT THE INVENTION

According to the present invention, the novel 3-epi-betulin-3-O-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside represented by the following formula (I) is isolated by a method which comprises grinding Pulsatillae radix, extracting the ground Pulsatillae radix with acetone, extracting again the acetone extract with hexane, filtering the extract to remove the filtrate, extracting again the residue (extract A) with water, subjecting the water-insoluble extract (extract B) to silica gel column chromatography eluting with water-saturated ethyl acetate to obtain the extract C, and then recrystallizing the extract C from methanol:

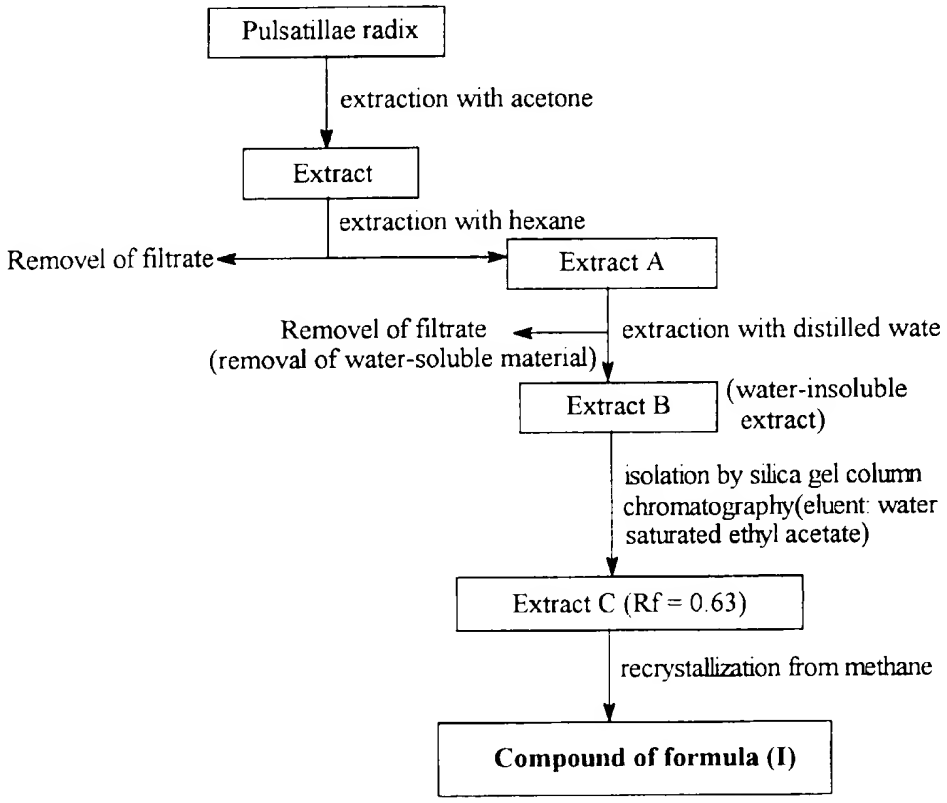


(I)

The novel compound of formula (I) exhibits a superior anti-cancer activity against solid tumors as demonstrated by the experimental results sepcifically described below. In addition, from the experimental results, it has also been disclosed that the purified extract of Pulsatillae radix obtained during the procedure for isolation and purification of the compound of formula (I), i.e.

the extracts A and B, can be effectively used as an anti-cancer agent. Therefore, the anti-cancer composition containing the extracts A and B as the active ingredient is also included in the present invention.

The process for extraction and isolation of the compound of formula (I) as mentioned above can be shown in the following flow chart:



The process of the present invention as mentioned above will be more specifically explained hereinafter.

In the first step, fresh Pulsatillae radix is extracted with acetone at a low temperature of 0-4℃. In this step, acetone is used in an amount of 1 to 10 parts by weight, preferably 3 to 7 parts by weight, with respect to one part by weight of fresh Pulsatillae radix. In view of the extraction efficiency, it

is particularly preferable that the extraction prodcedure with acetone is carried out by means of a cutting extractor which can carried out grinding and extracting procedures at the same time. In order to maximize the extraction efficiency with acetone, the residue isolated by filtering the extract can be repeatedly subjected to the same extraction procedure, preferably one to three times.

After the extraction is completed, the extract is filtered. In the second step, the filtrate is dried under reduced pressure to obtain the extract which is then extracted with hexane under agitating. For this purpose, hexane is suitably used in an amount of 10 to 50 parts by weight, preferably 20 to 30 parts by weight, with respect to one part by weight of the dried extract. In order to maximize the extraction efficiency with hexane, the residue isolated by filtering the extract can be repeatedly subjected to the same extraction procedure, preferably one to three times.

After the extraction is completed, the extract is filtered to remove the hexane extract. In the third step, the residue (extract A) is extracted with water, preferably with distilled water, and then filtered to recover an insoluble material. In this step, as water it is preferable to use warmed water, preferably water warmed to 30 to 50℃, in an amount of 1 to 20 parts by weight, preferably 5 to 15 parts by weight, with respect to one part by weight of the extract A. In addition, in order to maximize the extraction efficiency with water, the residue isolated by filtering the extract with warmed water can be repeatedly subjected to the same extraction procedure, preferably one to three times. Then the combined water solution was cooled to room temperature.

After the water-insoluble residue (extract B) isolated by filtration of water extract is dried, in the fourth step, the extract B is subjected to silica gel column chromatography eluting with water-saturated ethyl acetate to isolate the fraction of which the Rf value is identified as 0.63 by silica gel thin layer

chromatography, as the extract C. Then, the extract C is recrys- tallized from methanol to obtain the desired compound of formula (I).

The novel triterpene glycoside compound of formula (I) thus obtained exhibits a potent anti-cancer activity against cancers, particularly solid tumors, as mentioned above. Accordingly, the present invention relates to an anti-cancer composition comprising the compound of formula (I) as an active ingredient.

In addition, the extracts A and B obtained during the procedure for isolation of the compound of formula (I) can also exhibit a potent anti-cancer activity as demonstrated by the following experiments. Therefore, the present invention also includes an anti-cancer composition comprising the extracts A and B as the active ingredient.

When the anti-cancer composition comprising the compound of the present invention or the extract A or B is used for clinical purpose, it can be formulated into a conventional preparation in the pharmaceutical field, for example, preparation for oral administration such as tablet, capsule, troche, solution, suspension, etc., injectable preparation such as injectable solution or suspension, ready-to-use injectable dry powder which can be reconstituted with distilled water for injection when it is injected, etc., or topical preparation such as ointment, cream, solution, etc., by combining with a carrier conventionally used in the pharmaceutical field.

Suitable carrier which can be used in the composition of the present invention includes those conventionally used in the pharmaceutical field, for example, binder, lubricant, disintegrant, excipient, solubilizer, dispersing agent, stabilizing agent, suspending agent, coloring agent, perfume, etc. for oral preparation; preservative, pain alleviating agent, solubilizing agent, stabilizing agent, etc. for injectable preparation; and base, excipient, lubricant, preservative, etc. for topical preparation. The pharmaceutical preparation thus prepared can

be administered orally or parenterally, for example, intravenously, subcutaneously, intraperitoneally or topically. In addition, in order to prevent the active component from the decomposition with gastric acid, the oral preparation can be administered together with an antacid or in the enteric-coated form of the solid preparation such as tablet.

The dosage of the novel triterpene glycoside compound of formula (I) according to the present invention for human being can be suitably determined depending on absorption, inactivation and secretion of the active ingredient in the human body, age, sex and condition of subject patient, kinds and severity of cancer to be treated. Generally, in view of the experimental results, it may be preferable to administer the compound of formula (I) in an amount of 1 to 60㎎ per day for adult patient. However, it should be understood that depending on the judgement of specialists who supervise and monitor administration of the preparation or the individual requirement, the active compound of formula (I) can be administered in an amount beyond the dosage range as mentioned above. In addition, the composition of the present invention may be administered according to dosage regimen specialized in the cancer chemotherapy or in a multiple-divided dose via several times, preferably one to 6 times, at regular intervals.

The present invention is more specifically explained by the following examples. However, it should be understood that the present invention is not limited to these examples in any manner.

Example 1

200g of fresh radix of *Pulsatilla koreana* Nakai was introduced into a 1000㎖ cutting extractor and then ground with 700g of acetone at 4℃ for about 10 minutes while adjusting the rotation of cutting extractor to 2000rpm. The extract was filtered and the residue was extracted again with acetone according to the same procedure and then filtered. The combined filtrate was dried to obtain the powder of extract. The obtained powder was extracted

with 50㎖ hexane and then filtered to remove the filtrate. The remaining insoluble residue was dired to obtain 1.2g of the extract powder (extract A). 30㎖ of water was added to the obtained extract A and the mixture was stirred for 10 minutes at 40℃, cooled to room temperature and then filtered to obtain the insoluble residue which was dried to obtain 540㎎ of the extract (extract B). The silica gel thin layer chromatography of the extracts A and B is shown in Figure 1 as attached hereto.

Example 2

100g of silica gel (70-250 mesh) was introduced into a 250㎖ erlenmeyer flask and 20g of water was slowly added thereto and mixed together. The mixture was mixed again with water-saturated ethyl acetate and then poured into a column having diameter of 1.5㎝. The column was then stabilized by adding water-saturated ethyl acetate until the water-saturated ethyl acetate reaches the bottom of silica gel column.

500㎎ of the extract B obtained in Example 1 was mixed with 3g of silica gel (70-270 mesh) containing 20% water and then added to the column prepared above. Chromatography was carried out using water-saturated ethyl acetate as an eluent. The fractions were separately taken in an amount of 3 ㎖ and divided on the basis of Rf value as determined by silica gel thin layer chromatography [developing solvent: butanol/water/acetic acid=4/5/1, instant Kieselgel plate made by Merck Co.]. The fractions having Rf value of 0.63 (see Figure 1) were combined, dried and recrystallized from methanol to obtain 132㎎ of the crystal [material (I)].

The properties of the isolated material (I) are as follows.

Melting point : 226.2-228.1℃
UV (methanol solution) : no absorption band at 200㎚ or above
IR (KBr) : 3400cm^{-1}(br, OH), 2940(br, C-H), 1630(C=C), 1000-1100(C-O)

H-NMR (pyridine-d_5, ppm) : 6.215(1H, s, anomeric proton H-1''), 5.108(1H, d, J=6.4Hz, anomeric proton H-1'), 4.5-4.7(CH-O, m), 4.24-4.27(2H, m, CH_2O-C), 3.58 and 3.74(2H, dd, CH_2OH), 1.73(3H, s, 5. CH_3-C=C), 1.51(3H, d, J=6.1Hz), 0.99-1.16(4 · CH_3), 0.85(1 × CH_3)

Mass : molecular weight(M) 750, 604=[M-146]

In order to identify the structure of the material (I) as obtained above, the material (I) was subjected to hydrolysis or partial hydrolysis as follows.

Experiment 1 : Hydrolysis of the material (I)

30㎎ of the crystal obtained in Example 2 was dissolved in 2㎖ of 2N-sulfuric acid in 50% ethanol and then reacted under reflux for 2 hours. After the reaction is completed, the reaction solution was cooled, diluted with 3㎖ of water, neutralized with 2% $NaHCO_3$ solution and then extracted three times with 30㎖ of ether. The ether solution was isolated, dried with anhydrous sodium sulfate and then subjected to silica gel thin layer chromatography to monitor the degree of hydrolysis. The solvent was removed from the ether extract and the residue was then recrystallized from ether/ hexane. NMR of the resulting product was measured.

H-NMR of hydrolysate ($CDCl_3$, ppm): δ 4.7 and 4.6(2 × H, C=CH_2), 3.71 and 3.42(2 × H, dd, CH_2OH), 3.62(1 · H, t, CH-O at C-3), 1.69(3 × H, s, C=C-CH_3), 0.85-0.98(5 × CH_3)

From the above NMR data, the resulting hydrolystae was identified as 3-epi-betulin.

The aqueous layer which remains after isolation of the ether extract was dried and then extracted with methanol. The methanol-soluble portion was concentrated to approximately 1㎖ (water-soluble portion) and then subjected to thin layer chromatography to identify that the materials contained

in the aqueous layer are rhamnose and glucose. From these results, it could be identified that the material (I) before hydrolysis has the structure wherein 3-epi-betulin is combined to sugars such as glucose and rhamnose.

Experiment 2 : Partial hydrolysis of the material (I)

9.0㎎ of the material (I) obtained in Example 2 was dissolved in 5㎖ of 0.2N sulfuric acid in 50% ethanol and refluxed for 2 hours. The degree of hydrolysis was monitored by silica gel thin layer chromatograph using the material (I) as the comparative substance at a given period. After the reaction is completed, the reaction solution was neutralized with potassium carbonate and extracted with ethyl acetate, and the extract was then dehydrated with anhydrous sodium sulfate. On silica gel thin layer, three spots corresponding to the starting material (I), aglycon and the material having middle Rf value between Rf values of material (I) and aglycon were markedly shown. In order to isolate the material having middle Rf value, the hydrolysate was extracted with water-saturated ethyl acetate in silica gel column containing 20% water. The isolated material (material M) was dried and then subjected to mass spectroscopy.

Mass spectrum of material M : $[M^+]$=604, $[M^+-163]$=441

From the mass spectrum data, it was identified that the material M is 3-epi-betulin-3-glucoside.

According to the analytical data of hydrolysate and partial hydrolysate, it is evident that the material (I) is a 3-rhamnosyl-glucoside of 3-epi-betulin. The NMR spectrum of the material (I) says that the glucosyl moiety is bound to 3-OH of 3-epi-betulin in β-position and C-1 of the rahmnosyl moiety is bound to C-6 of the glucosyl moiety.

The structure of the material (I) is determined to be 3-epi-betulin-α

-L-rahmnopyranosyl (1→6)-β-D-glucopyranoside having the formula (I) above.

Experiment 3 : Test for anti-cancer activity of the material (I) against S-180 solid tumor

In this experiment, healthy male ICR mouse weighing 18 to 22g which were obtained from Korean Experimental Animal Center were used as a test animal. The selected test animals were given water and feed without any restriction in the chamber controlled at a temperature of 23-24℃. As the feed, the commercial anitibiotic-free feed for mouse was used.

S-180 cells were incubated for 7 days within peritoneal cavity of ICR mouse for incubation of S-180 cells and isolated together with ascites. To the isolated cells was added sterilized cold physiological saline and the mixture was centrifuged with 400×g for 2 minutes to isolate the cell precipitate. The isolated cell precipitate was suspended again in cold physiological saline and then centrifuged to remove the supernatant. Only S-180 cells were taken, excluding any red blood cells incorporated therein, washed three times with the same method as above, and then suspended to obtain the cell suspension in concentration of 10^7 cells/㎖ by counting the number of cells with a hemacytometer. ICR mouse for the test, which were obtained from Korean Experimental Animal Center were anesthetized by exposing them to ether vapor. Between both shoulders of the test animal, each 0.1㎖ of the suspension of S-180 cancer cells as prepared above was subcutaneously injected. After 5 days from transplantation of cancer cells, mice in which cancer was induced were selected and then divided so that each group contains 10 mouse. To the control group was injected 0.1㎖ of PEG200 (polyethyleneglycol 200) per day and to the test group was injected 0.1㎖ of the solution of the test drug in PEG200, for 5 days. In each test group, the extract A and B obtained in Example 1 was continuously administered in an amount of 0.3㎎/㎏, 0.5㎎/㎏, 1.0㎎/㎏, 1.5㎎/㎏ or 2.0㎎/㎏ per day for 5 days, and the compound of formula (I) obtained in Example 2 [material (I)]

was intraperitoneally injected in an amount of 0.01㎎/㎏, 0.1㎎/㎏, 1.0㎎/㎏ or 10㎎/㎏ per day for consecutive 5 days.

10 days after administration of the test drug, i.e. 20 days after transplantation of cancer cells, the tumor mass was isolated from the test animal and then weighed. The inhibition rate for cancer was calculated by the following equation:

$$\text{Inhibition rate}(\%) = (C-T) \times 100/C$$

In the above equation, C means the weight (g) of the tumor mass in the control group and T means the weight (g) of the tumor mass in the test group.

According to the above equation, it was determined that the extract A shows the maximum inhibition rate of 87% at dosage of 1.5㎎/㎏ and the extract B shows the maximum inhibition rate of 91% at dosage of 0.5㎎/㎏. In addition, it was also determined that the compound of formula (I) [material (I)] shows the inhibition rate of 84% at dosage of 0.01㎎/㎏, 96% at dosage of 0.1㎎/㎏, 90% at dosage of 1.0㎎/㎏, and 35% at dosage of 10㎎/㎏. That is, it could be identified that the compound of formula (I) shows the best inhibition rate at dosage of 0.1㎎/㎏ and the inhibition rate decreases at dosage of 10㎎/㎏ or more.

From the above experimental result, it is apparent that the compound of formula (I) according to the present invention and the extracts A and B exhibit a potent growth inhibiting activity against S-180 cancer cells as solid tumor, and therefore, can be very effectively used as an anti-cancer agent.

Experiment 4 : Acute toxicity test

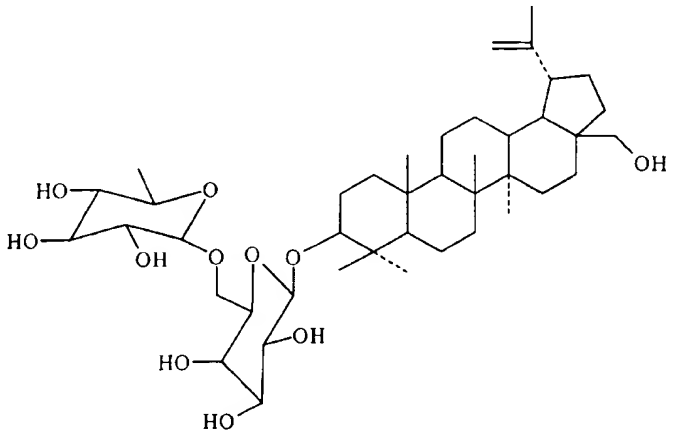
As the test animal 5 male and 5 female mouse weighing 20 to 25g

were used to determine the acute toxicity of triterpene glycoside of formula (I) according to the present invention.

The test animal was given via oral route the compound of formula (I) suspended in 1㎖ of physiological saline in the maximum amount of 500㎎/㎏ and then observed for 14 days. No death was observed. Accordingly, it could be identified that the novel triterpene glycoside of formula (I) according to the present invention has substantially no toxicity at the therapeutic dosage range.

WHAT IS CLAIMED IS :

1. Triterpene glycoside compound represented by the following formula (I):



(I)

2. A process for preparing the extract A of Pulsatillae radix which comprises grinding Pulsatillae radix, extracting the ground Pulsatillae radix with acetone, extracting again the acetone extract with hexane, filtering the extract to remove the filtrate and recovering the residue.

3. A process for preparing the extract B of Pulsatillae radix which comprises extracting the extract A of Pulsatillae radix obtained according to the method of claim 2 with hexane and water in sequence and filtering the extract to recover the insoluble material.

4. A process for preparing triterpene glycoside of formula (I) as defined in claim 1 which comprises subjecting the extract B of Pulsatillae radix obtained according to the method of claim 3 to silica gel column chromatography eluting with water-saturated ethyl acetate to obtain the extract C and recrystallizing the extract C from methanol.

5. An anti-cancer composition which comprises the extract A of Pulsatillae radix obtained according to the method of claim 2 as an active ingredient.

6. An anti-cancer composition which comprises the extract B of Pulsatillae radix obtained according to the method of claim 3 as an active ingredient.

7. An anti-cancer composition which comprises triterpene glycoside of formula (I) obtained according to the method of claim 4 as an active ingredient.

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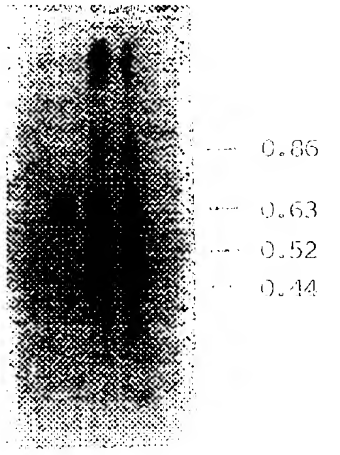
Fig. 1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00232

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 07 H 15/256; A 61 K 31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 07 H; A 61 K

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CAS, WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Vol.114, No.19, 13 May 1991 (Columbus, Ohio, USA), page 450, column 1, abstract No.182070a, YE, W. et al.:"Chemical constituents of Pulsatilla chinensis (I)", Zhongguo Yaoke Daxue Xuebao 1990, 21(5), 264-6 (Ch).	1-7
A	Chemical Abstracts, Vol.125, No.5, 29 July 1996 (Columbus, Ohio, USA), page 634, column 2, abstract No.53565u, YE, W. et al.:"Triterpenoids from Pulsatilla chinensis", Phytochemistry 1996, 42(3), 799-802 (Eng).	1-7

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